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REMARKS

Claims 1, 3-8, 11, 13-16, 18-22, 28-31, 36-44, 46, 48-66, 68-72, 74, 76-85, 88, 91-95, 97-100, and 133-137 are currently pending. Claims 4, 28, 50, 56, 94, 133-134, and 136 are amended herein. Support for the amendments is found throughout the application, and therefore, it is believed that no new matter has been added. Claims 31 and 97 are canceled herein. Claims 1, 3-8, 11, 13-16, 18-27, 29-30, 36-44, 46, 48-49, 51-53, 55, 57-66, 68-74, 76-85, 88, 91-95, and 98-100 are allowable.

Formal Matters

Applicants gratefully appreciate the withdrawal of some of the previous rejections and objections, and the indication that claims 1, 3-8, 11, 13-16, 18-27, 29-30, 36-44, 46, 48-49, 51-53, 55, 57-66, 68-74, 76-85, 88, 91-95, and 98-100 are allowable.

Objection

The Examiner objects to claim 4, asserting that the term "plant cell" in the thereby clause of claim 4 should be replaced with "plant" for clarification. Applicants have amended claim 4 herein per the Examiner's suggestion. Therefore, Applicants respectfully request the withdrawal of this objection.

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 28, 50, 54, 56, 97, and 133-137 are rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite. According to the Examiner, claim 28 is indefinite for (1) depending upon cancelled claim 23 and (2) containing the limitation "metabolic pathway enhances an input or output trait in a plant cell" as the metes and bounds of this limitation is allegedly unclear. Claims 50 and 56 are rejected because the term "plant cells" lack antecedent basis. The Examiner assert that claim 54 does not further limit claim 52. Claims 94 and 97 are rejected because the term "genetically modified plant cell" lacks antecedent basis. The Examiner also points out that claim 97 depends on canceled claim 87. The Examiner also argues that claims 133-34 and 136-37 are indefinite because the term "derived" is unclear. The Examiner suggests substituting the term "obtained" for the term "derived." Applicants traverse this rejection.

Applicants respectfully submit that claim 28 as amended herein fulfills the definiteness requirement under 35 U.S.C. § 112, second paragraph. First, claim 28 as amended properly depends on claim 1. Second, the metes and bounds of the claimed invention are apparent to the ordinary artisan. The Examiner asserts that the terms "input trait" and "output trait" are so broad that one of ordinary skill in the art cannot determine the metes and bounds of the invention. However, the breadth of a claim alone is insufficient to render a claim in definite. *See* MPEP § 2173.04 ("Breadth of a claim is not to be equated with indefiniteness." (citations omitted)). The terms at issue are terms of art as evidenced by the dictionary definitions and examples already on record. The ordinary artisan would understand that input traits are those that increase production efficiency while output traits enhance the quality of the target product. *See, e.g.*, Exhibit A (discussing the use of biotechnology techniques to enhance input and output traits in various crops). Applicants note that the Examiner has provided no evidence that the meaning of the terms of art at issue are not clear to the ordinary artisan. As the terms are an art recognized terms and Applicants are using the terms as they are used in the art, the terms "input trait" and "output trait" are sufficiently definite.

Claims 50 and 56 are amended herein to provide proper antecedent basis for "plant cell."

Applicants respectfully submit that claim 54 properly further limits claim 52. Claim 52 relates to the modulation of gene expression by at least two fold. Claim 54 relates to the modulation of gene expression where the modulation is at least two fold activation. Therefore, claim 54 further limits the modulation of gene expression by defining the modulation as an activation of gene expression. Thus, claim 54 is properly dependent on claim 52.

Claim 94 is amended herein to indicate that the plant cell is a stably transformed plant cell.

Claim 97 is canceled herewith.

Claims 133, 134, and 136 are each amended to substitute the term "obtained" for the term "derived." Applicants believe that the term "derived" is sufficiently clear when read in light of the independent claim. Nonetheless, the claim is amended herewith simply to expedite prosecution of the instant application.

In view of the above, Applicants respectfully submit that the basis for the rejection may be withdrawn.

Rejection Under 35 U.S.C. § 112, First Paragraph - Enablement

Claims 28, 31, and 55 remain rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to be fully enabled by the instant specification for reasons of record. Briefly, the Examiner asserts that the specification lacks reasonable enablement for methods employing an expression vector encoding a synthetic zinc finger protein to stably modulate gene expression of a target gene where the target gene encodes a protein that controls a metabolic pathway that enhances an input or output trait in a plant cell or the modulation changes the phenotype of the plant. With regards to claim 31, the Examiner alleges the specification provides no guidance for identifying a disorder associated with abnormal gene expression in plant cells or how to treat such diseases. Applicants traverse this rejection.

Applicants respectfully submit that the working examples and the guidance provided by the specification reasonably enables the use of the claimed methods to modulate a target gene to change the phenotype of a plant cell and the resultant plant. Applicants provide a working example demonstrating the feasibility of the using synthetic zinc fingers to modulate the phenotype of a plant. *See, e.g.*, Example 8. In this example, the phenotype of the stably transformed plant is clearly altered. In fact, the specification describes three different phenotypes that resulted from the stable expression of the synthetic zinc finger protein. *See, e.g.*, the specification at page 98 (describing significant phenotypic changes in flower structures and fertility). This data definitively shows that synthetic zinc finger proteins can be stably expressed and result in phenotypic change.

Additional post-filing data provides further support for the stable expression of synthetic zinc finger proteins and their resultant phenotypic changes. *See* Exhibit B and C. In Exhibit B, a synthetic zinc finger protein that modulates AP3, a transcription factor that determines floral organ identity, is stably expressed. The authors demonstrate that synthetic zinc fingers can manipulate transgene and endogenous gene expression without affecting gene expression in non-targeted genes, that this manipulation of gene expression resulted in phenotypic changes, and that the expression of the synthetic zinc finger expression was heritable. *See* Exhibit B. In Exhibit C, objective evidence demonstrates the range of the activity of the synthetic zinc fingers in regulating gene expression. Using three different promoter constructs, the authors demonstrate that synthetic zinc fingers successfully and stably upregulate genes controlled by the targeted promoters. Moreover, the data

presented in Exhibit C also demonstrates unequivocally the stability of heritability of the synthetic zinc finger gene in plant cells. The data shown in Exhibit B and C use the guidance and methods as provided in the specification as filed. In fact, some of the data shown in Exhibit B is from the same experiments as disclosed in Example 8. *See* Exhibit 8 at, *e.g.*, page 13299 (regarding data for plant cell ND0052-2e). Therefore, these references provide further objective evidence that the specification fully enables the claimed methods.

Applicants submit that the disclosed data and additional objective evidence provided herein provide reasonable enablement for the claimed method to stably modulate gene expression where the modulation results in phenotypic change in the plant. The Examiner appears to be asserting that Applicants must reduce to practice every embodiment of the claimed methods. However, there is no requirement that every gene that can be modulated by this method be demonstrated. For a claimed genus, representative examples together with a statement applicable to the genus as a whole will ordinarily be sufficient if the ordinary artisan would expect the claimed genus could be used in a that manner without undue experimentation. MPEP § 2164.02. Applicants have provided representative examples, a statement applicable to the use of the claimed methods with the genus of genes that can be regulated by synthetic zinc fingers, as well as objective evidence demonstrating the sufficiency of the guidance in the specification. *See* the specification at page 43, line 2 (statement of applicability to genus). Moreover, the claimed methods build on the knowledge of the art regarding the well known properties of zinc fingers and their role in gene regulation. The changing of a phenotype by modulation of gene expression is well known in the art, and therefore, the demonstration that synthetic zinc finger proteins can alter a phenotype is sufficient for the ordinary artisan to extend the guidance and working examples in the specification to other zinc finger-regulated genes.

Contrary to the Examiner's assertion that no evidence has been presented that gene regulation by synthetic zinc finger proteins results in phenotypic changes, the specification provides actual working examples where synthetic zinc finger proteins change the phenotype of the plant. *See* Example 8. If the Examiner does not believe the data, Applicants request a clear statement as to the deficiencies of the data presented. Applicants also note that phytic acid is not encoded by a gene

as apparently believed by the Examiner, but rather phytic acid is the product of a synthetic pathway in which MIPs participates.

Likewise, the specification provides reasonable enablement for the claimed method where the modulation of gene expression enhances an input or output trait in a plant. As discussed above, these traits are readily identifiable by the ordinary artisan. Manipulations to enhance input and output traits are already routinely performed in the art using various methods of recombinant DNA technology. *See, e.g.*, Exhibit A. Thus, in providing working examples and the necessary protocols to use the claimed methods employing synthetic zinc finger proteins, the specification provides the ordinary artisan sufficient guidance to enhance input and output traits.

While Applicants believe the specification fully enables claim 31, the claim is canceled herewith in order to expedite the prosecution of the instant application, thereby rendering the rejection moot. Applicants expressly reserve their right to pursue the canceled subject matter in a related application.

In view of the above, Applicants respectfully submit that the basis for the rejection may be withdrawn.

Rejection Under 35 U.S.C. § 112, First Paragraph - Written Description

Claims 28, 30, 31, 55, and 70-71 remain rejected under 35 U.S.C. § 112, first paragraph as lacking sufficient written description. According to the Examiner, the specification provides no description of a method to stably modulate the expression of a target gene encoding a protein that controls a metabolic pathway that enhances an input or output trait in the plant cell or plant or where the modulated gene expression changes the phenotype of a plant cell or plant. The Examiner rejected claims 30 and 70-71, alleging that the genes encoding a cofactor and a phytic acid are not described in the specification or the prior art. Applicants traverse this rejection.

Applicants respectfully submit the specification clearly conveys to the ordinary artisan that Applicants had possession of the claimed method at the time of filing. The invention lies in the ability to use synthetic zinc finger proteins to modulate gene expression *in vivo* in a plant cell and the resulting plant, not in the identification of novel genes or metabolic pathways or novel phenotypic changes. Thus, the specification provides adequate written description for these claims.

It is well known in the art that genes encode proteins that function in metabolic pathways and that the alteration of gene expression results in phenotypic changes. In fact, it is likely the ordinary artisan would be hard pressed to identify a gene that did not participate in a metabolic pathway in some way or whose alteration ultimately does not result in some phenotypic change. Moreover, claim 28 as currently amended does not require that the protein necessarily be identified as part of a metabolic pathway, thus the specification provides adequate written description for these claims.

While Applicants maintain that the specification provides adequate written description to support claim 30, it is canceled herein simply to expedite the prosecution of the instant application.

Finally, Applicants submit that the specification adequately described the co-factor and phytic acid of claims 70-71 without providing genes encoding the co-factor or phytic acid. First, as previously discussed, phytic acid is synthesized by a metabolic pathway, and thus is not encoded by a genetic sequence. Second, the term "co-factor" is a term of art exemplified by KRAB, MAD, and others that encompasses enzymatic co-factors. *See* the specification at page 38, lines 14-19. Again, the invention lies not in the identity of a novel enzyme co-factor, but the application of the claimed methods to regulate the expression of such co-factors. Thus, the specification provides sufficient written description for the claimed invention.

In view of the above, Applicants respectfully submit that the basis for the rejection may be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding objection and rejections of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 278012001420. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: June 9, 2004

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Biotechnology Offers US Farmers Promises And Problems

Gregg Hillyer
Progressive Farmer

Transgenic crops promise to drastically change how farmers produce and market their feedgrains and oilseeds. These changes will produce challenges for United States (US) farmers and the entire agricultural industry. How these obstacles are resolved will impact the future use of biotechnology on US farms.

Key words: agriculture; biotechnology; farmers; genetically modified crops (GMC); input traits; output traits, transgenic

Biotechnology promises to add another chapter to the revolutionary changes that have shaped US agriculture over the past 100 years. Just like the switch from horses to horsepower and mechanical weed control to chemical control, genetic engineering will forever change how farmers produce crops. But unlike previous breakthroughs, biotechnology may rewrite the book on production agriculture—and the entire industry.

What determines whether or not a farmer adopts a new technology? Basically, it comes down to two simple questions. First, does it work? And second, will it make money? From a farmer's perspective, technology is successful only if it is

profitable.

To find out, farmers generally try a promising new technology on a limited basis first. If they see a clear advantage and are comfortable using it, they expand acreage until it becomes a standard production scheme on their farms. This adoption process usually occurs over several years, through steady and sustained growth.

But acceptance of biotechnology down on the farm is occurring at an unprecedented growth rate. In 1995, there were no commercial plantings of genetically modified crops (GMCs) in the US Today, it is estimated that 33% of the corn crop, 44% of the soybean acres, and 55% of cotton fields are planted to transgenic hybrids and varieties that have built-in resistance to selected insects and herbicides. Industry watchers expect the bio-engineered expansion to continue as new products come on the market.

The First Wave Of Biotechnology

Input traits represent the first wave of biotechnology. They provide a new level of protection against pests and are a powerful weapon in weed control arsenals. Early commercial products include *Bacillus thuringiensis* (Bt) corn. These genetically-altered hybrids contain a naturally-occurring soil bacterium, Bt, that kills European corn borers. Bacillus thuringiensis cotton protects the crop against tobacco budworm and bollworm. Farmers can expect to see genetically engineered corn hybrids that resist rootworms in the next two to three years.

To fight weeds, farmers have several genetically-engineered options to choose from. Roundup Ready (glyphosate-tolerant) soybeans and corn, and LibertyLink (glufosinate ammonium) corn are some examples. These crops are immune to the broad spectrum, but non-selective herbicides, such as Roundup, Touchdown

and Liberty. When applied, the herbicide kills the weeds without harming the crop. In the case of cotton, farmers can turn to BXN (Bromoxynil) or Roundup Ready herbicide-tolerant varieties. More herbicide-resistant crops are on the way.

The rapid adoption of biotechnology can be attributed to several factors. These are as follows:

- *Cost savings.* Herbicide- and insect-resistant crops generally lower pesticide use and require fewer trips across the field. Fewer trips means lower energy costs.
- *Convenience.* Most new technology is easy to use. Farmers are also well familiar with a herbicide like Roundup and know what to expect in weed control performance. In addition, broad spectrum control requires less time to scout and manage crops.
- *Environmentally friendly.* Less pesticides reduce the odds of potential runoff. And many of the GMOs encourage the use of conservation tillage practices, enhancing efforts to save soil and to protect water quality.

Companies plan to add more value to bioengineered crops by stacking multiple traits. Several products are already available for corn and cotton that provide herbicide- and insect-tolerance. In addition, scientists are searching to unlock the genetic codes that will protect crops against environmental stresses (droughts, floods, and temperatures) and to enhance a crop's ability to utilize nutrients and water resources.

That is good news for farmers. Biotechnology will offer them additional tools to protect—and increase—yield potential. But while the benefits of these

biotechnology crops are readily apparent, farmers are still sorting out how they fit into their overall operation. Questions they continue to ponder include the following:

- *Do GMCs pay?* High-tech seed carries premium prices. Some companies attach a technology fee of $5 to $15 per acre to purchase the seed. That is in addition to the higher prices this seed generally carries versus conventional choices. Farmers must factor in these added costs when making purchasing decisions.

  Insect pressure is difficult to predict and may not warrant the use of an insect-tolerant variety every year. If it is planted under low insect conditions, the genetically altered seed becomes expensive insurance against the threat of insect damage. Also, variable growing conditions between fields and regions make it nearly impossible to formulate projected economic returns on a consistent basis.

- *Do GMCs yield?* There are no yield guarantees regardless of the kind of seed you plant. However, when the first herbicide-resistant soybeans were released, for example, many farmers reported that they did not yield as well as the elite non-GMC varieties. Some thought the new technology produced a yield drag. But in truth, it was because many of the early GMCs were not placed in a company's elite seed. As old germplasm is replaced with the newest and the best, concerns over yield differences—real or perceived—will decrease.

  When first released, transgenic cotton also experienced growing pains in certain growing regions. The yield-hampering problems were amplified by environmental stresses and other factors. In both instances, farmers learned valuable lessons: GMCs are not a cure-all. And some may require

a higher level of management than conventional crops.

- *Will GMCs last?* The overuse of any technology fuels fears that weeds or insects may eventually develop resistance to the science. That's one reason farmers are required to plant a certain percentage of their acres to a refuge (non-GMC hybrid) when using Bt corn. For herbicide-tolerant crops, rotating herbicides and mode of actions will help reduce the odds of resistant weeds.

- *Who will buy GMCs?* The European Union and some Asian countries view biotechnology, and food derived from the gene-altered crops, with hostility and suspicion. This viewpoint threatens potential grain sales for US farmers. If there is not a market for a crop, farmers are not going to plant it. The fallout from Europe's concerns was amplified earlier this year when some US processors announced that they would not buy corn from hybrids not approved by the European Union.

The Second Wave Of Biotechnology

The second wave of biotechnology is output traits. Unlike input traits that are designed to protect and enhance yield, output traits promise to enhance the value of the crops from the farmer to the consumer. A myriad of specialized grains with unique uses is, and soon will be, on the market. For growing tailored traits, farmers can earn premiums on each bushel. These premiums range from as little as 20 cents per bushel to several dollars per bushel depending on market demand and specifications. Dr. Charles Hurburgh, a specialist in grain quality at Iowa State University, predicts that 40% of the corn and soybeans grown in the US will eventually contain a value-added trait for a specific end use.

Early efforts in valued-added crops have focused on enhancing the value of animal feed since livestock are the dominant users of feedgrains. This has led to the development of high-oil corn and hybrids with increased levels of amino acids and starch, to name a few. Other traits include low phytate corn, also known as high available phosphorus corn, that increases the digestibility of the phytate nutrient by swine and poultry. As a result, less phosphorus is excreted in the manure, making it more environmentally friendly.

For soybeans, many of the tailored traits are being developed to produce healthier oils and soy foods. The most common specialty soybeans are high oleic, high sucrose, low saturate, low linolenic and low null (produces a less beany taste).

Work is also progressing to turn plants into factories, using bio-engineered crops for renewable energy sources and industrial uses. Genetic engineering may also help tailor plants into nutraceuticals—the blending of a regular food product with a health-enhancing attribute, like calcium-enriched orange juice.

Finally, some see biotechnology as a way to use plants to produce vaccines and other important medicines. Or the crop could be manipulated to contain a drug allowing it to be distributed and administered orally.

Like their input trait cousins, specialty crops that contain bioengineered output traits also face potential challenges that will impact the rate of adoption by farmers. These challenges are as follows:

- *Do not fool Mother Nature.* A living plant can throw any number of roadblocks at science, no matter how good the technology. What may make sense in the laboratory many not make sense to the plant. When you ask a plant to do something different from its normal physiology, you

do not know how it will react. So the promise of something big could turn instead into an empty promise.

- *Share the wealth.* Everyone who participates in a value-added system must be adequately compensated. Farmers worry premiums may disappear if everyone jumps in. There is also concern they will not share in the higher margins that generally go to players outside the farm gate.
- *Build solid relationships.* To be a viable player in the value-added chain, farmers must develop relationships along the entire chain. Specialty grains could eventually change the entire scope of production agriculture, moving it towards contract production, similar to what is found in the poultry and hog industry. Interdependence will replace independence. Some farmers may choose not to participate.
- *Keep it separate.* Specialty grains must be segregated from commodity crops. The question is who will develop and pay for the immense infrastructure required for value-added grains? Costs could be so prohibitive that the returns may not justify a farmer's participation.
- *Politics.* Our trading partners overseas are much more skeptical of genetically modified crops than we are in the US. These hurdles will be difficult, but not impossible, to overcome. Acceptance will be slow. Regardless, consumers everywhere must believe there is value in the final product no matter what the level of technology.

Like most revolutions, no one really knows how it will end. But one thing is certain, biotechnology offers exciting promises and prospects for the American farmer. And it will forever change US agriculture.

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Heritable endogenous gene regulation in plants with designed polydactyl zinc finger transcription factors

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Contributed by Steven P. Briggs, July 10, 2002

Zinc finger transcription factors (TFs_{ZF}) were designed and applied to transgene and endogenous gene regulation in stably transformed plants. The target of the TFs_{ZF} is the *Arabidopsis* gene *APETALA3* (*AP3*), which encodes a transcription factor that determines floral organ identity. A zinc finger protein (ZFP) was designed to specifically bind to a region upstream of *AP3*. *AP3* transcription was induced by transformation of leaf protoplasts with a transformation vector that expressed a TF_{ZF} consisting of the ZFP fused to the tetrameric repeat of herpes simplex VP16's minimal activation domain. Histochemical staining of β-glucuronidase (GUS) activity in transgenic AP3::GUS reporter plants expressing GUS under control of the *AP3* promoter was increased dramatically in petals when the *AP3*-specific TF_{ZF} activator was cointroduced. TF_{ZF}-amplified GUS expression signals were also evident in sepal tissues of these double-transgenic plants. Floral phenotype changes indicative of endogenous *AP3* factor coactivation were also observed. The same *AP3*-specific ZFP^{AP3} was also fused to a human transcriptional repression domain, the mSIN3 interaction domain, and introduced into either AP3::GUS-expressing plants or wild-type *Arabidopsis* plants. Dramatic repression of endogenous *AP3* expression in floral tissue resulted when a constitutive promoter was used to drive the expression of this TF_{ZF}. These plants were also sterile. When a floral tissue-specific promoter from *APETALA1* (*AP1*) gene was used, floral phenotype changes were also observed, but in contrast the plants were fertile. Our results demonstrate that artificial transcriptional factors based on synthetic zinc finger proteins are capable of stable and specific regulation of endogenous genes through multiple generations in multicellular organisms.

floral development | *APETALA3* | *APETALA1*

In nature, eukaryotic nuclear genes are tightly regulated at both the transcriptional and translational levels. Much of this control is achieved through DNA-binding transcription factors. The manipulation of plant traits in agricultural biotechnology would be greatly facilitated if preselected endogenous genes could be turned on or off in a controlled and selective manner. A conceptual approach to such manipulation is the engineered expression of specific native transcription factors that have evolved to control particular genes. Advances in whole-genome sequencing of *Arabidopsis* (1) and more recently rice (2), combined with informatics-based analysis have allowed the identification of numerous putative plant transcription factors (2, 3). However, the identification and characterization of the molecular targets of these transcription factors is still at a very early stage, and consequently it is not yet possible to use them broadly as gene-specific tools for controlled regulation of endogenous gene expression. Rational design of artificial transcription factors that target specific DNA sequences with non-native nucleotide binding domains fused to transcriptional activation or repression domains is therefore an attractive option. An especially promising approach of this kind utilizes synthetic DNA binding domains of the zinc finger protein (ZFP) class.

Numerous zinc finger DNA-binding domain motifs have been identified in genes originating from plants and other biological systems (4–7). Among these, the Cys-2–His-2 type of ZFP has been the subject of the most extensive structural, biochemical, and genetic studies (4–24). This highly modular zinc finger domain has been found to be particularly amenable to rational manipulation of target binding site specificity. Several design and selection strategies have been developed for construction of synthetic zinc finger-based DNA binding proteins that can be highly specific for given target sequences (8, 10–24). Recently, several studies have demonstrated targeting of endogenous genes in cultured mammalian cells using synthetic ZFP-based artificial transcription factors where the DNA binding domain of the ZFP has been fused to transcriptional activation or repression domains (18–24). Application of this technology to agriculture by means of designing plant-specific zinc finger transcription factors (TFs_{ZF}) would potentially enable a range of diverse applications. However, two critical issues remain to be addressed: the function and stability of TFs_{ZF} in a multicellular organism that has been regenerated from a transformed cell, and the ability of these genes to be stably inherited in subsequent generations.

In this study, we describe artificial transcription factors based on a designed polydactyl ZFP that specifically targets the *AP3* floral development gene of *Arabidopsis thaliana*. Wild-type *Arabidopsis* flowers have four organ types (sepal, petal, stamen, carpel) arranged in concentric whorls (25). *AP3*, a member of the MADS box gene family (26, 27), is involved in specifying the organ identity of floral whorl 2 (petal) and whorl 3 (stamen) (28–30). Altered expression of *AP3* results in homeotic mutations where whorl-specific organ identity is perturbed. Severe *ap3* mutant alleles and mutations in the related gene *pistillata* cause organ identity changes and sterility (31, 32). Such readily observed phenotypes make the *Arabidopsis* flower a useful system in which to study manipulated gene regulation. A suitable target site for ZFP design using GNN repeat motifs (16–19) is located approximately 50 bp upstream of the *AP3* TATA box. A six-finger ZFP with predicted specificity for this 18-bp site was designed and synthesized (19). A TFs_{ZF} for gene activation was prepared by fusion of the synthetic tetrameric repeat of herpes simplex VP16's minimal activation domain (VP64) to the designed ZFP. For gene-specific repression, a mammalian repression domain, mSin3 interaction domain (Sid), was fused to the ZFP (19, 21). Transformation of these TFs_{ZF} into *Arabidopsis* yielded transgenic phenotypes similar to known *ap3* mutant alleles. Introduction of *AP3*-specific TFs_{ZF} into *Arabidopsis*

Abbreviations: ZFP, zinc finger protein; TFs_{ZF}, zinc finger transcription factors; VP64, tetrameric repeat of herpes simplex VP16's minimal activation domain; Sid, mammalian mSin3 interaction domain; GUS, β-glucuronidase; ZFP^{AP3}, AP3-specific zinc finger protein.

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plants expressing a β-glucuronidase (GUS) reporter gene under control of the *AP3* promoter resulted in GUS expression changes reflective of the various promoters used to drive TFs_{ZF} expression. Our results demonstrate that artificial transcription factors based on engineered ZFPs can be used to manipulate endogenous transgene gene expression in multicellular organisms in both a transient and stable fashion.

Materials and Methods

Plant Materials and Promoters. A 1.9-kb *AP1* promoter sequence and transgenic plants expressing GUS under control of the *AP3* promoter originally generated by Tom Jack's laboratory (25) was obtained from Detlef Weigel (The Salk Institute, La Jolla, CA).

Construction and Characterization of AP3-Specific ZFP^{AP3}. A ZFP was synthesized to bind to the complementary strand of the18-bp sequence 5′-TACTTCTTCAACTCCATC-3′ found at −112 to −95 relative to the start of translation of the *APETALA3* genomic sequence (33). The gene was constructed and the protein expressed and purified as a fusion with maltose binding protein as described. ELISA specificity and electrophoretic mobility shift assays were performed as described.

Construction of Zinc Finger-Effector Domain Fusions and Transformation of Plants. TFs_{ZF} bearing the VP64 activation domain and the Sid repression domain were prepared as described (19). For constitutive expression, the TFs_{ZF} were cloned into a dicot expression vector pNOV102 (34) downstream from the UBQ3 promoter (35, 36) and upstream of the nos transcriptional terminator. The resulting constructs (UBQ3::ZFP^{AP3}-VP64//nos and UBQ3::Sid-ZFP^{AP3}//nos) were transformed into *A. thaliana* plants (Columbia) using the agrobacteria-mediated transformation method (37). Protoplast transient transformation and assay were conducted as described (34). Putative transgenic plants were selected for hygromycin resistance as described (34). A nontarget activation construct UBQ3::ZFP^{m4}-VP64//nos was generated and transformed into *Arabidopsis* at the same time. ZFP^{m4} targets the sequence of maize *myo*inositol 1-phosphate synthase. ZFP^{m4}-VP64 activates transcriptions of *myo*inositol 1-phosphate synthase in maize cells (34). A transformation control vector UBQ3::GFP//nos was used for all transformations. For floral tissue-specific regulation, a 1.9-kb fragment containing the *AP1* promoter was substituted for the UBQ3 promoter fragment. The resulting constructs (AP1::ZFP^{AP3}-VP64//nos and AP1::Sid -ZFP^{AP3}//nos) were transformed into *Arabidopsis* as described above.

RT-PCR Analysis of AP3 Expression Level. Plant tissues (either protoplasts or floral tissue from stages 1 to approximately stage 15; ref. 38) were collected and frozen immediately in liquid nitrogen. Total RNA was prepared from these samples by using the Ambion RNAwiz kit (Ambion). The expression levels of the ZFP artificial transcription factors and endogenous *AP3* were monitored by RT-PCR. RT-PCRs were carried out in 25-μl volumes by using 200 ng of RNA and the Qiagen 1-step RT-PCR kit. *AP3*-specific primers were Ap3-F, 5′-GGCGAGAGGGAAGATCCAG-3′ and Ap3–4R, 5′-CTCCTCTAATACGACTCACTATAGGGACACTCACCTAGCCTCTG-3′. The thermocycler settings were 50°C for 30 min, 95°C for 15 min (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) × 30 cycles, and 72°C for 10 min.

Quantitative PCR Analysis of *AP3* Expression Level. Total RNA used for RT-PCR was assayed quantitatively on an ABI Prism 7900 Sequence Detector (Taqman) according to the manufacturer's instructions (Beckman Coulter). All probes and primers were designed with the program PRIMER EXPRESS with the default setting (Perkin–Elmer). For *AP3* detection, the probe sequence 5′-CCATTTCATCCTCAAGACGACGCAGCT-3′ was used with primers 5′-TTTGGACGAGCTTGACATTCAG-3′ (forward) and 5′-CGCGAACGAGTTTGAAAGTG-3′ (reverse). Taqman PCRs were carried out by using 250 ng total RNA and Taqman one-step RT-PCR master mix reagent (Perkin–Elmer). Thermal cycling conditions were 48°C for 30 min, 95°C for 10 min for 1 cycle, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression was quantified by using the comparative Ct method with the thioredoxin gene TRX3 as an internal expression standard. The probe sequence used for TRX3 was 5′-AGACTTCACTGCAACATGGTGCCCAC-3′ with primers 5′-GTGTGGAAATGACACAGATTGTGA-3′ (forward) and 5′-AGACGGGTGCAATGAAACG-3′ (reverse).

GUS Histochemical Staining and Analysis of Expression Pattern. GUS histochemical staining was conducted as described (39, 40). Freshly excised floral tissues were immersed immediately in GUS staining solution containing 0.25 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylammonium salt (X-glu, Rose Scientific, Edmonton, AB, Canada) in 50 mM sodium phosphate buffer (pH 7.3), and then incubated 20 h at 37°C. Stained tissues were dehydrated in an ethanol series and photographed before infiltration with Histo-Clear (International Diagnostics, Atlanta), and paraffin-cast specimen blocks (41) were sectioned on a microtome (Microm HM315, Mikron) and stained *in situ* with 0.1% aqueous Safranin-O for 5–60 s. Stained surfaces of specimen blocks were treated with immersion oil and visualized under a dissecting microscope (Olympus, New Hyde Park, NY).

Results

Synthesis and Characterization of ZFP. The target sequence of the designed *AP3*-specific ZFP^{AP3} is an 18-bp sequence located in the *AP3* promoter region, at −112 to −95 relative to the ATG codon (Fig. 1*A*). This six-domain ZFP was assembled from zinc finger domains of predefined specificity as described (16, 17, 19, 23). ZFP^{AP3} was expressed in *Escherichia coli* and purified. ZFP^{AP3} demonstrated good specificity in ELISA assays using a panel of oligonucleotide target sequences (data not shown). The affinity of the ZFP^{AP3} for its designed target was determined to be 2.3 nM by electrophoretic mobility assays. This affinity is in the range we have previously determined to be required for endogenous gene regulation (21, 23).

Transient Activation of Endogenous *AP3* in *Arabidopsis* Leaf Cells. *AP3* is normally expressed exclusively in developing flowers, and no expression of the gene has been reported in leaf mesophyll protoplasts. Such protoplasts were used to investigate transcriptional activation of *AP3*. The *AP3*-specific activation construct UBQ3::ZFP^{AP3}-VP64//nos and a nontarget activation construct UBQ3::ZFP^{m4}-VP64//nos (which targets the sequence of maize *myo*inositol 1-phosphate synthase) were transformed into *Arabidopsis* leaf protoplasts. UBQ3::GFP//nos was used as a transformation control. All samples were analyzed by RT-PCR to determine the baseline levels of endogenous *AP3* transcripts. The *AP3*-specific primers were designed to yield a 600-bp product if amplification is based on a genomic DNA template (indicating contamination) and 334 bp for transcribed *AP3* messages. The originally silent *AP3* gene was activated in cells that were transformed with the *AP3*-specific activation construct UBQ3::ZFP^{AP3}-VP64//nos but not with any other constructs (Fig. 2). These results indicate that the ZFP^{AP3} domain is able to bind *AP3* DNA and direct the activation domain (VP64) to the specific and otherwise silenced endogenous target *in vivo*. Furthermore, TFs_{ZF} targeted to different genes (e.g., ZFP^{m4}) did not activate *AP3* in a nonspecific manner.

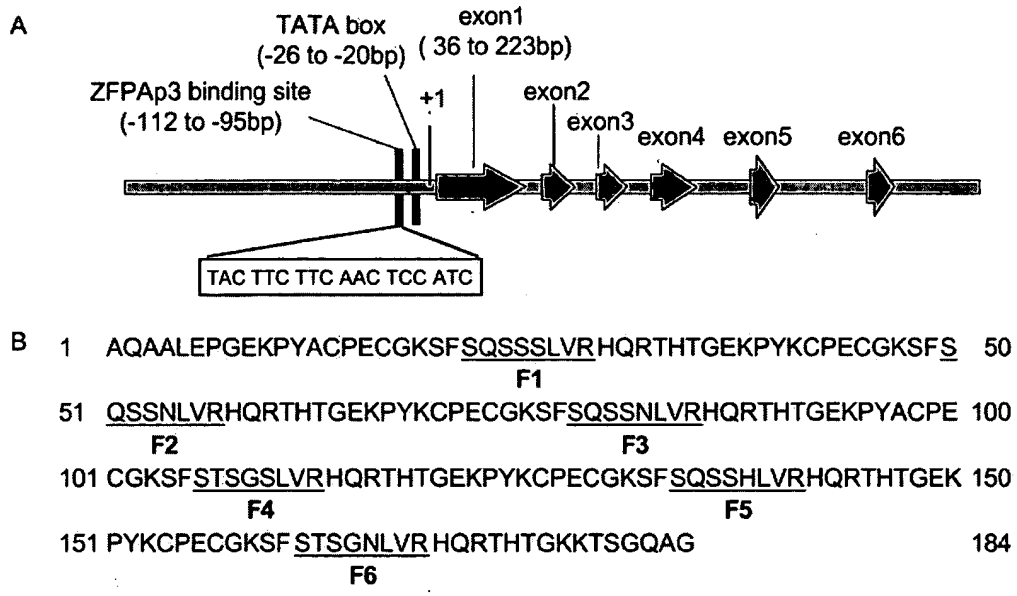

Fig. 1. (*A*) ZFP^{AP3} target sequence (boxed) and its position in the *AP3* 5′ UTR. Numbers indicate the distance from the ATG translation initiation codon. The arrowed boxes indicate the exon of *AP3*. (*B*) DNA recognition helix sequences of the ZFP^{AP3} protein. The underlined amino acids are the components of the new zinc fingers that provide specificity for the selected nucleotide sequences indicated in *A*. The recognition helices of fingers 1–6 (F1–F6) are underlined.

Phenotypic Consequences of *in Planta* Activation of *AP3*. Attempts to stably transform *Arabidopsis* with the construct UBQ3::ZFP^{AP3}-VP64//nos to constitutively activate *AP3* expression were inefficient. Few transformants could be obtained with this vector. Most had rearrangements that lacked the VP64 domain. None expressed ZFP^{AP3}-VP64. To circumvent this problem, we restricted *AP3* activation to floral tissue. For this, the *APETALA1* (*AP1*) promoter was used to direct expression of ZFP^{AP3}-VP64. To dissect *AP1*-specific regulation of *AP3* from the morphological consequence of altered *AP3* transcription factor activity, we examined the effects of AP1::ZFP^{AP3}-VP64//nos first in an *AP3*-driven GUS reporter background (AP3::GUS). This reporter has been characterized (25) and established a characteristic *AP3*-specific pattern of GUS staining. Floral tissue-specific activation construct AP1::ZFP^{AP3}-VP64//nos and control construct AP1::ZFP^{AP3}//nos were transformed into homozygous *Arabidopsis* plants harboring construct AP3::GUS. The expression of *AP3* starts from stage 3 of flower development and is accumulated in petal and stamen primordia only. As a result of this late expression, AP3::GUS plants accumulate a moderate level of the stable reporter in the petals and stamens, as illustrated in Fig. 3*A*. In contrast, *AP1* is expressed at the very early stages (stages 1–3) of flower development (42, 43), when the floral organ primordia are just beginning to initiate. This results in an *AP1* signal that is uniformly distributed throughout the young floral primordia and later in development is restricted to the sepal and petal primordia (Fig. 3*A*). Consequently, we predict that a plant double-transformed with constructs AP1::ZFP^{AP3}-VP64//nos and AP3::GUS will initiate *AP3*-directed expression at the very early stage of floral primordia development (stages 1–3), coinciding with *AP1* expression, and will also show the petal and stamen pattern at later stages of the characteristics of normal *AP3* expression. To test this hypothesis, flowers from many double transgenic plants were stained for GUS and observed either with or without tissue mounting. The GUS staining patterns shown in Fig. 3 revealed strong activation of the *AP3* promoter in an *AP1*-dependent manner as predicted: (*i*) the GUS signals expanded throughout the entire flower primordia, starting at the very earliest stages of development (Fig. 3 *C* and *E*); (*ii*) the GUS signal was detected in the sepals of mature flowers (Fig. 3 *C* and *E*); and (*iii*) the intensity of the GUS signal increased dramatically in the petals of mature flowers (Fig. 3 *C* and *E*). These patterns were distinct from the petal and stamen staining of the AP3::GUS line. In our system, both the transgenic GUS gene and the native *AP3* transcription factor gene should each be subject to *AP1*-dependent activation by the *AP3*-targeted TFs_{ZF}. Reflective of this, we found that several independent transgenic plants showed distinct phenotypic changes in floral development, suggesting an altered pattern of *AP3* transcription factor activity. In these plants, some whorl 1 organs are replaced by whorl 2 organs, resulting in flowers having five or more petals (Fig. 4*B*) and reduced or absent sepals. The stamens appear normal and all plants are fertile. An interesting floral phenotype was observed in some of the floral-specific activation events obtained using construct AP1:ZFP^{AP3}-VP64. These plants exhibited a markedly higher percentage of young flowers in the

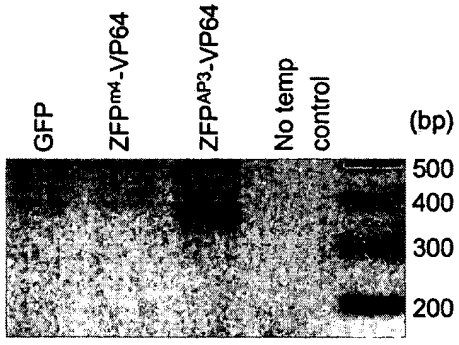

Fig. 2. Activation of the silenced endogenous *AP3* gene in *Arabidopsis* leaf cells. RT-PCR was used to detect *AP3* expression in *Arabidopsis* leaf protoplasts transformed with *GFP* (control), ZFP^{m4}-*VP64* (nonspecific activation control), and ZFP^{AP3}-*VP64* (*AP3*-specific activation). The last lane (−) is no template control amplification.

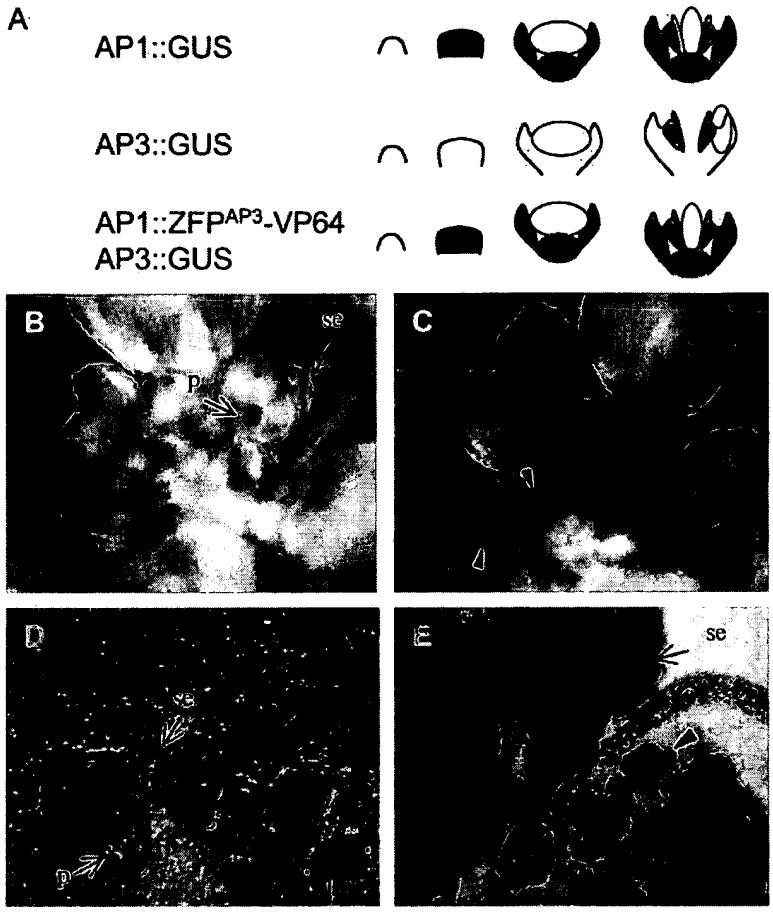

Fig. 3. GUS staining flowers of background plant with AP3::GUS only and double transgenic plant with both AP3::GUS and activation construct AP1::ZFP^{AP3}-VP64//nos. (*A*). Predicted GUS staining patterns of *AP3::GUS* and AP1::GUS (based on personal communication with Martin Yanofsky, University of California, San Diego). (*B*) Flowers from background plant with AP3::GUS only stained for GUS activity. GUS signal is detected only in the petal (p) and stamen (not visible here, see Fig. 6*A*), but not in the carpal (not visible) and sepal (se). Picture taken directly after staining procedure. (*C*) Flowers from double transgenic plant expressing AP3::GUS and AP1::ZFP^{AP3}-VP64//nos simultaneously. GUS signal is increased in petal and is detected throughout the young flower primordia. (*D*) Mounted flowers from background plant with AP3::GUS only stained for GUS activity. GUS signal is detected only in the petal (p) and stamen (not visible here, see Fig. 7*A*) and not in the carpal and sepal (se). (*E*) Mounted flowers from double transgenic plant expressing AP3::GUS and AP1::ZFP^{AP3}-VP64//nos simultaneously. GUS signal is increased in petal (p), extended to sepal (se), and detected throughout the young flower primordia (arrow).

inflorescence tissue as compared with controls (data not shown). Activation of the AP3::GUS transgene and the endogenous *AP3* gene were both found to be stable genetically and were transmitted faithfully to progeny over two subsequent generations (T3).

***In Planta* Repression of Endogenous AP3.** For *in planta* repression, a constitutive UBQ3 promoter was used to drive the expression of an *AP3*-specific ZFP fused to a human transcriptional repression domain (19). This construct (UBQ3::Sid-ZFP^{AP3}//nos) was transformed into wild-type *Arabidopsis*. We found that both TFs$_{ZF}$ and endogenous *AP3* expression levels varied over a wide range among independent transgenic events. However, endogenous *AP3* gene expression levels were repressed in most transgenic lines. Higher TFs$_{ZF}$ expression in line *ND0052–2e* (Fig. 5*A*) produced a marked down regulation of endogenous *AP3* expression (Fig. 5*B*). Quantitative RT-PCR analysis revealed a nearly 50-fold *AP3* repression in this line (data not shown). We also

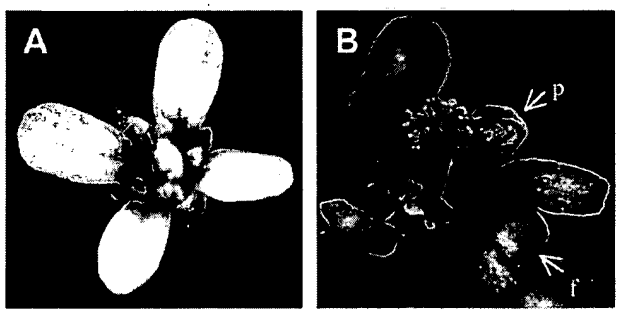

Fig. 4. Floral phenotypic changes in double transgenic plant expressing AP3::GUS and AP1::ZFP^{AP3}-VP64//nos simultaneously. A seven-petal flower is shown here. Two extra petals are fully converted (f), and the third one is partially converted (p).

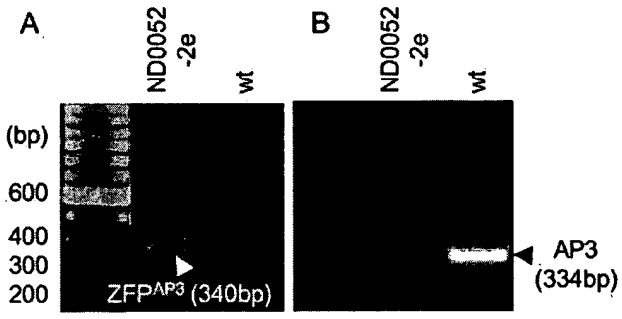

Fig. 5. Repression of endogenous *AP3* expression by the constitutive repression construct UBQ3::Sid-ZFP^{AP3}//nos in transgenic plant *ND0052–2e*. (*A*) RT-PCR identification of transgene *ZFP^{AP3}* in transgenic event *ND0052–2e* and wild-type control plant. (*B*) RT-PCR evaluation of endogenous gene *AP3* expression level in transgenic event *ND0052–2e* and wild-type plant. In plant *ND0052–2e*, the expression of *AP3* is significantly repressed by the expression of repressor *Sid-ZFP^{AP3}* fusion protein. Quantitative PCR indicated 46-fold repression.

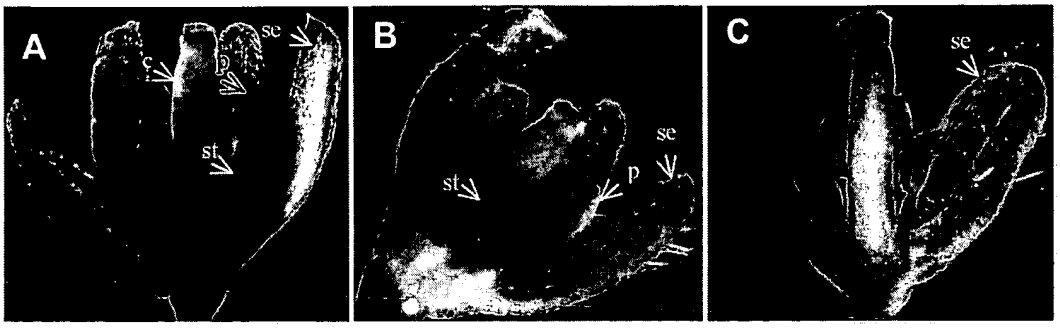

Fig. 6. GUS staining flowers of background plant with AP3::GUS only and double transgenic plant with both AP3::GUS and repression construct AP1::Sid-ZFP^{AP3}//nos. (*A*) Flowers from background plant with AP3::GUS only stained for GUS activity. GUS signal is detected only in the petal (p) and stamen (st) and not in the carpal and sepal (se). Picture taken directly after staining procedure. (*B*) Flowers from double transgenic plant expressing AP3::GUS and AP1::Sid-ZFP^{AP3}//nos simultaneously. GUS signal disappeared from petals but is detectable in stamens. (*C*) Flowers from a different double transgenic plant expressing AP3::GUS and AP1::Sid-ZFP^{AP3}//nos simultaneously. Low level GUS activity is detectable in stamens. In addition, the petals were absent in this flower.

observed that most of the transgenic plants having reduced *AP3* expression were sterile and had unopened flowers at maturity. Dissection of flowers from *ND0052–2e* plants revealed petals that were shorter and narrower than wild-type flowers of the same maturity. In addition, the lower portions of the petals were converted partially to sepal-like structures, and the stamens were greatly reduced in size compared with wild type. The floral phenotypes of the strongly *AP3*-repressed plants are similar to the flowers of the characterized *ap3* and *sap* mutants (44, 45).

Floral tissue-specific repression, the construct AP1::Sid-ZFP^{AP3}//nos and control construct AP1::ZFP^{AP3}//nos, lacking a repression domain, were transformed into *Arabidopsis* plants already carrying an AP3::GUS (25) transgene stably integrated into the genome. Because *AP1* is expressed in whorls 1 and 2 (sepals and petals), whereas *AP3* is expressed in whorls 2 and 3 (petals and stamens), we predicted that the GUS signal should be eliminated from whorl 2 but not whorl 3 in the double transgenic plant-expressing construct AP1::Sid-ZFP^{AP3}//nos in the presence of AP3::GUS. Fig. 6 shows this expected pattern of stamen-only GUS expression. Petals from some of these flowers are absent, as expected if the endogenous *AP3* gene is repressed (Fig. 6*C*). Two types of petal morphology were observed in these double transformants: missing petals (Figs. 6*C* and 7*C*) and partial conversion of petals to sepals (sepaloid petals) (Fig. 7*B*). These plants were fully fertile despite the floral alterations. Only the constitutive expressed ZFP^{AP3} plants were sterile.

Discussion

We have shown that ZFP-based artificial transcription factors can be designed and synthesized to manipulate transgene and endogenous gene expression levels in transgenic plants. Although regulation of only one transgene (GUS) and one endogenous gene (*AP3*) are presented here, we believe this approach will be generally applicable to all genes. This study supports and extends previous studies of TFs$_{ZF}$ that have been designed to activate and repress endogenous genes in mammalian cells (8, 21–24). Therefore, we believe that this approach should be viable in other transgenic organisms as well.

Our studies indicate that transcriptional activation and repression with our TFs$_{ZF}$ are specific for the targeted *AP3* gene. Preliminary GeneChip (47) analysis of transgenic plants with or without ZFP^{AP3}-effector fusion revealed no significant changes in gene expression in the 8,000 nontargeted floral or nonfloral genes examined (data not shown). Despite this apparent specificity, recovery of plant lines that constitutively expressed TFs$_{ZF}$ activators (UBQ3::ZFP^{AP3}-VP64//nos) was inefficient. In addition, we observed that the VP64 tetrameric repeat was subject to somatic rearrangement in plant cells. This result parallels difficulties encountered in the generation of transgenic animals and cell lines expressing the VP16 activation domain or designed versions of this domain like VP64, suggesting that this activation domain itself carries with it an intrinsic toxicity (48).

We believe that our TFs$_{ZF}$ approach to gene regulation can be further enhanced by combination with other gene regulation

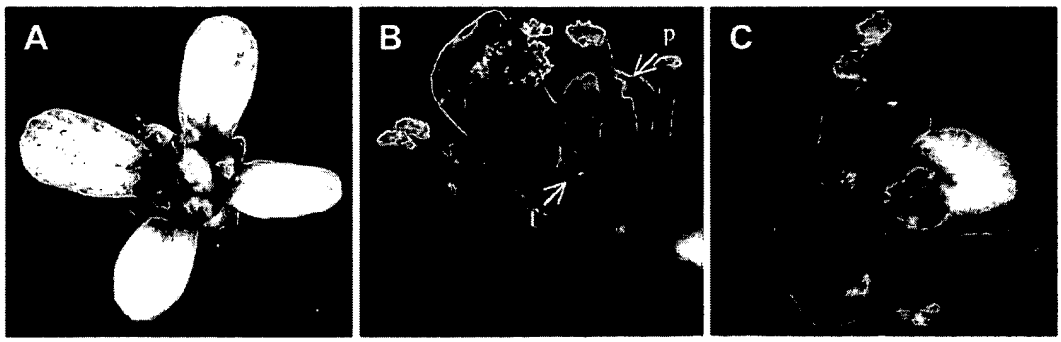

Fig. 7. Floral phenotypic changes in double transgenic plant expressing AP3::GUS and AP1::Sid-ZFP^{AP3}//nos simultaneously. (*A*) Flowers from background plant with AP3::GUS only. (*B*) Flowers from double transgenic plant expressing AP3::GUS and AP1::Sid-ZFP^{AP3}//nos simultaneously. A three-petal flower is shown here. In this flower, an extra sepal is fully replaced by one petal (f), and another petal is partially replaced (p). (*C*) Flowers from a different double transgenic plant expressing AP3::GUS and AP1::Sid-ZFP^{AP3}//nos simultaneously with one missing petal.

technologies, such as inducible gene expression, which has been recently adapted to create chemically regulated TFs_{ZF} (49). Tissue-specific expression of the TFs_{ZF} provides for another level of control as demonstrated here. Jack and colleagues have shown that overexpression of *AP3* causes conversion of sepals to petals, carpels to stamens, as well as loss of fertility (32, 44). Presumably, tissue-specific activation of *AP3* in our *AP1*-driven activation plants allows them to maintain their fertility. Likewise, transcriptional repression with the human Sid domain in plants was potent and yielded phenotypes analogous to those observed in plants harboring a temperature-sensitive allele of *AP3* (46), suggesting that both gain of function and loss-of-function phenotypes are accessible by using zinc finger technology in whole organisms.

Note Added in Proof. In a companion article, we have demonstrated the efficacy of our TF_{ZF} approach in transgenic tobacco plants maintained over several generations (50).

1. The *Arabidopsis* Genome Initiative (2000) *Nature (London)* **408,** 796–815.
2. Goff, S., Ricke, D., Lan, T., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., *et al.* (2002) *Science* **296,** 92–100.
3. Riechmann, J., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O. J., Samaha, R. R., *et al.* (2000) *Science* **290,** 2105–2110.
4. Berg, J. M. (1990) *Annu. Rev. Biophys. Biophys. Chem.* **19,** 405–421.
5. Ashworth, A. & Denny, P. (1991) *Mamm. Genome* **1,** 196–200.
6. Pieler, T. & Bellefroid, E. (1994) *Mol. Biol. Rep.* **20,** 1–8.
7. Takatsuji, H. (1998) *Cell. Mol. Life Sci.* **54,** 582–596.
8. Beerli, R. & Barbas, C. F., III (2002) *Nat. Biotechnol.* **20,** 135–141.
9. Elrod-Erickson, M., Rould, M. A., Nekludova, L. & Pabo, C. O. (1996) *Structure (London)* **4,** 1171–1180.
10. Desjarlais, J. R. & Berg, J. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7345–7349.
11. Wu, H., Yang, W. & Barbas, C. F., III (1995) *Proc. Natl. Acad. Sci. USA* **92,** 344–348.
12. Rebar, E. J. & Pabo, C. O. (1994) *Science* **263,** 671–673.
13. Choo, Y. & Klug, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11163–11167.
14. Wolfe, S. A., Grant, R. A., Elrod-Erickson, M. & Pabo, C. O. (2001) *Structure (London)* **9,** 717–723.
15. Greisman, H. A. & Pabo, C. O. (1997) *Science* **275,** 657–661.
16. Segal, D. J., Dreier, B., Beerli, R. R. & Barbas, C. F., III (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2758–2763.
17. Dreier, B., Segal, D. J. & Barbas, C. F., III (2000) *J. Mol. Biol.* **303,** 489–502.
18. Liu, Q., Segal, D. J., Ghiara, J. B. & Barbas, C. F., III (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5525–5530.
19. Beerli, R. R., Segal, D. J., Dreier, B. & Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14628–14633.
20. Kang, J. S. & Kim, J. (2000) *J. Biol. Chem.* **275,** 8742–8748.
21. Beerli, R. R., Dreier, B. & Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* **97,** 1495–1500.
22. Zhang, L., Spratt, S. K., Liu, Q., Johnstone, B., Qi, H., Raschke, E. E., Jamieson, A. C., Rebar, E. J., Wolffe, A. P. & Case, C. C. (2000) *J. Biol. Chem.* **275,** 33850–33860.
23. Dreier, B., Beerli, R., Segal, D., Flippin, J. & Barbas, C. F., III (2001) *J. Biol. Chem.* **276,** 29466–29478.
24. Liu, P., Rebar, E. J., Zhang, L., Liu, Q., Jamieson, A. C., Liang, Y., Qi, H., Li, P. X., Chen, B., Mendel, M. C., *et al.* (2001) *J. Biol. Chem.* **276,** 11323–11334.
25. Weigel, D. (1995) *Annu. Rev. Genet.* **29,** 19–39.
26. Theissen, G. & Saedler, H. (1995) *Curr. Opin. Genet. Dev.* **5,** 628–639.
27. Bowman, J. L., Baum, S. F., Eshed, Y., Putterill, J. & Alvarez, J. (1999) *Curr. Top. Dev. Biol.* **45,** 155–205.
28. Yanofsky, M. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 167–188.
29. Irish, V. F. (1999) *Dev. Biol.* **209,** 211–220.
30. Jenik, P. D. & Irish, V. F. (2001) *Development (Cambridge, U.K.)* **128,** 13–23.
31. Jack, T., Brockman, L. L. & Meyerowitz, E. M. (1992) *Cell* **68,** 683–687.
32. Jack, T., Fox, G. L. & Meyerowitz, E. M. (1994) *Cell* **76,** 703–716.
33. Irish, V. F. & Yamamoto, Y. T. (1995) *Plant Cell* **7,** 1635–1644.
34. Barbas, C. F., III, Stege, J., Guan, X. & Dalmia, B. (2001) PCT WO 01/52620.
35. Norris, S. R., Meyer, S. E. & Callis, J. (1993) *Plant Mol. Biol.* **21,** 895–906.
36. Callis, J., Raasch, J. A. & Vierstra, R. D. (1990) *J. Biol. Chem.* **265,** 12486–12493.
37. Bechtold, N. & Pelletier, G. (1998) in *Arabidopsis Protocols*, eds. Martinez-Zapater, J. & Salinas, J. (Humana, Clifton, NJ), pp. 259–266.
38. Bowman, J., ed. (1993) in *Arabidopsis, An Atlas of Morphology and Development* (Springer, New York), pp. 135–145.
39. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) *EMBO J.* **6,** 3901–3907.
40. Sessions, A., Weigel, D. & Yanofsky, M. (1999) *Plant J.* **20,** 259–263.
41. Ruzin, S. E., eds. (1999) in *Plant Microtechnique and Microscopy* (Oxford Univ. Press, New York), pp. 57–86.
42. Mandel, A. M., Gustafson-Brown, C., Savidge, B. & Yanofsky, M. F. (1992) *Nature (London)* **360,** 273–277.
43. Gustafson-Brown, C., Savidge, B. & Yanofsky, M. F. (1994) *Cell* **76,** 131–143.
44. Byzova, M. V., Franken, J., Aarts, M. G., de Almeida-Engler, J., Engler, G., Mariani, C., Van Lookeren Campagne, M. M. & Angenent, G. C. (1999) *Genes Dev.* **13,** 1002–1014.
45. Yi, Y. & Jack, T. (1998) *Plant Cell* **10,** 1465–1477.
46. Ren, D., Collingwood, T. N., Rebar, E. J., Wolffe, A. P. & Camp, H. S. (2002) *Genes Dev.* **16,** 27–32.
47. Zhu, T. & Wang, X. (2000.) *Plant Physiol.* **124,** 1472–1476.
48. Akagi, K., Kanai, M., Saya, H., Kozu, T. & Berns, A. (2001) *Nucleic Acids Res.* **29,** 1–5.
49. Beerli, R. R., Schopfer, U., Dreier, B. & Barbas, C. F., III (2000) *J. Biol. Chem.* **275,** 32617–32627.
50. Ordiz, M. I., Barbas, C. F., III, & Beachy, R. N. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 13290–13295.

Regulation of transgene expression in plants with polydactyl zinc finger transcription factors

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Designer zinc finger transcription factors (TFs_{ZF}) have been developed to control the expression of transgenes and endogenous genes in mammalian cells. Application of TFs_{ZF} technology in plants would enable a wide range of both basic and applied studies. In this paper, we report the use of TFs_{ZF} to target a defined 18-bp DNA sequence to control gene expression in plant cells and in transgenic plants. A β-glucuronidase reporter gene was activated by using the designed six-zinc finger protein 2C7 expressed as a fusion with the herpes simplex virus VP16 transcription factor activation domain. Reporter gene expression was activated 5- to 30-fold by using TFs_{ZF} in BY-2 protoplasts, whereas expression was increased as much as 450 times in transgenic tobacco plants. Use of a phloem-specific promoter to drive expression of the TFs_{ZF} resulted in activation of the reporter gene in vascular tissues. Transgenic tobacco plants that produce 2C7 transcription factors were phenotypically normal through two generations, suggesting that the factors exerted no adverse effects. This study demonstrates the utility of zinc finger technology in plants, setting the stage for its application in basic and applied agricultural biotechnology.

Contemporary models of eukaryotic gene expression recognize the dynamic mechanisms and pathways that, in the end, determine whether or not a gene is expressed and to what level. Mechanisms that regulate gene expression include chromatin modification and remodeling, binding of multiple factors to DNA cis elements, binding of the RNA polymerase, transcription elongation, termination, and posttranscriptional regulation. The interactions between protein factors and DNA sequences at or near the gene promoter are key components in gene regulation and depend on the accessibility of the region in the context of chromatin (1).

A variety of technical approaches have been used to control gene expression experimentally. One common approach involves the use of synthetic or chimeric genes and transcription factors (2). For example, the yeast galactose inducible Gal4 DNA-binding protein has been fused with activation or repression domains to create previously uncharacterized transcription factors that can act on target genes that incorporate the Gal4 operator sequence (3–5). Recently, synthetic transcription factors based on the assembly of multiple zinc finger domains have been developed for similar purposes. Zinc finger transcription factors are among the most common transcription factors and there may be as many as 700 genes that encode such factors in the human genome. It is estimated that *Arabidopsis* contain 85 genes that encode zinc finger transcription factors (6). Such synthetic zinc fingers transcription factors (TFs_{ZF}) can be custom designed for binding to any DNA sequence (7, 8).

A single zinc finger typically binds three nucleotides, and "polydactyl" fingers comprising multiple zinc fingers have been developed to bind unique DNA sequences and to regulate gene expression (7, 8). Six-finger proteins bind 18 contiguous nucleotides, are predicted to be highly specific for the sites to which they bind, and can potentially be used to regulate expression of genes in many types of organisms. A number of studies have shown that TFs_{ZF} can activate or repress the expression of transgenes or endogenous genes in mammalian cells (7–11).

We initiated the study described herein to determine whether TFs_{ZF} can be used to regulate gene expression in plant cells. We used a well characterized polydactyl six-zinc finger protein, referred to as 2C7, and its cognate 18-bp 2C7 DNA-binding sequence (11), in plant protoplasts and in transgenic plants. In these studies we found that binding of the 2C7 protein increased the expression of three different core promoters. Furthermore, expression in both protoplasts and transgenic plants increased when the herpes simplex virus VP16 activation domain was added to the zinc finger protein. In transgenic plants that contain the zinc finger protein and a reporter gene construct, we observed a >450-fold induction of gene expression. These studies support the conclusion that TFs_{ZF} can be a powerful tool to regulate gene expression in plants and will be useful both in plant genomics and plant biotechnology.

Experimental Procedures

Construction of Plasmids. Promoter fragments from Cassava Vein Mosaic Virus (CsVMV) (12) designated as D (nucleotides −178 to +72), E (nucleotides −112 to +72), and F (nucleotides −63 to +72) were subcloned in the pBluescript II KS(+) vector (Stratagene) and fused to the *uidA* gene (pILTAB402, pILTAB403, and pILTAB404, respectively). Plasmids C7D::G, C7E::G, and C7F::G were constructed by inserting six copies of the 2C7 recognition site from the previously described pGL3 promoter construct (11) via restriction sites *Mlu*I and *Bgl*II. These sites are 5′ of the D, E, and F fragments of the CsVMV promoter between *Xba*I and *Bam*HI restriction sites. All gene constructs were confirmed by DNA sequencing (Fig. 1*A*). The T fragment (nucleotides −32 to +45) of the promoter from Rice Tungro Bacilliform virus (RTBV) was ligated to six tandem repeats of the 2C7-binding sites into the *Xba*I site upstream of the fragment (C7T::G). The T fragment contains the TATA box of RTBV promoter plus nucleotides to +45 (ref. 13; Fig. 2*A*). Plasmid C7er2::G contains the minimal promoter of the erbB-2 gene (9). In this plasmid the *Luc* gene was replaced by the *uidA* gene with the nos terminator sequence by using the restriction sites *Nco*I and *Kpn*I (Fig. 2*A*).

To obtain the expression plasmids that produce the effector proteins (TFs_{ZF}), the ORF encoding the polydactyl protein 2C7 (11), with the transcription activator domain VP16 (11) or VP64 (9) were excised with *Bam*HI and *Hin*dIII from pcDNA3 (11). The fragment was subcloned in *Bgl*II and *Eco*RI sites of a plasmid, which contains the enhanced 35S promoter from cauliflower mosaic virus (e-35S) promoter (14) and the nos terminator sequences (Figs. 1*A* and 2*A*).

To produce the reporter gene for transgenic plants, we subcloned the C7F::G construct (Fig. 3*A*), with *Not*I and *Hin*dIII, into the *Bam*HI site of pGJ3571. The TFs_{ZF} constructs were removed from their respective plasmids with *Not*I and placed in

Abbreviations: GUS, β-glucuronidase; CsVMV, Cassava Vein Mosaic virus; RTBV, Rice Tungro Bacilliform virus; TFs_{ZF}, zinc finger transcription factor; e-35S, enhanced 35S promoter; MU, 4-methylumbelliferone.

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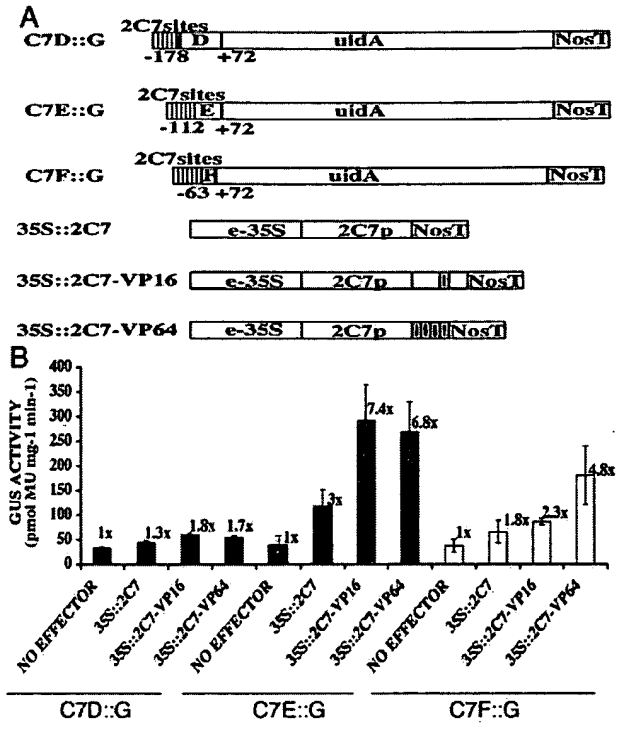

Fig. 1. Regulation of GUS expression in BY-2 protoplasts using fragments of the CsVMV promoter with and without the TFs_{ZF} effector genes. (*A*) Diagram of the reporter and effector constructs. Different lengths of the CsVMV promoter, D (nucleotides −178 to +72), E (nucleotides −112 to +72), and F (nucleotides −63 to +72; ref. 12) with the 2C7-binding sites in a 5′ position relative to the TATA sequence, were ligated with the *uidA* gene. The enhanced 35S promoter was ligated to coding regions of the 2C7 TFs_{ZF} protein, or the 2C7 protein fused at the C-terminal region with the herpes simplex virus transcription activation domain, VP16 or VP64. (*B*) Measurement of GUS activity in extracts of protoplasts 24 h after DNA transfection. The protoplasts were cotransfected with a mixture of DNAs including 5 μg of reporter construct DNA, 20 μg of TFs_{ZF} effector construct DNA, 5 μg of the 35S::Luc plasmid DNA, and 10 μg of herring sperm DNA. The results are expressed as the amount of GUS enzyme activity in picomol 4-methylumbelliferone (MU) mg^{-1}·min^{-1}. The results are the average of three independent experiments, three samples per experiment ± SD. The results of assays that contain the effector genes are significantly different from samples containing only the correspondent reporter construct ($P < 0.05$).

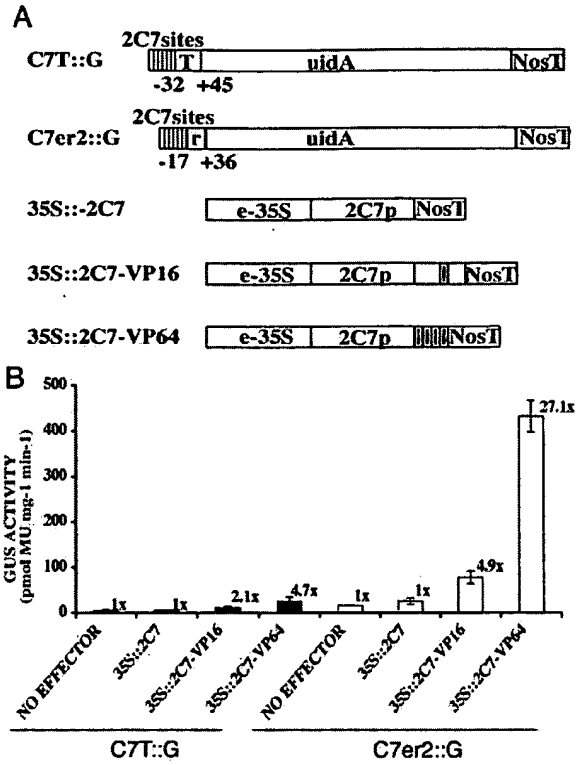

Fig. 2. Expression of GUS activity in BY-2 protoplasts using the T fragment of the RTBV promoter and a minimal human erbB-2 promoter. (*A*) Diagram of the reporter and effector constructs used. T fragment (nucleotides −32 to +45) of RTBV and the minimal promoter of erbB-2 (er2; nucleotides −17 to +32) with the 2C7 recognition sites in the 5′ region were fused with the *uidA* reporter sequence (C7T::G and C7er2::G). The effector constructs are described in Fig. 1. (*B*) Quantification of GUS activity from extracts 24 h after transfection. Protoplasts were cotransfected with a mixture of DNAs including 5 μg of reporter construct C7T::G or C7er2::G, 20 μg of TFs_{ZF} effector construct, 5 μg of the 35S::GFP plasmid, and 10 μg of herring sperm DNA. Results are expressed as amount of GUS enzyme activity as picomol MU mg^{-1}·min^{-1} compared to GFP activity. The results are the average of three independent transfections ± SD ($P < 0.05$).

the *Hin*dIII site in the polylinker of the *Agrobacterium tumefaciens* vector pGJ357. The vector pGJ357 is a modification of the plasmid pPZP200 (15). In plasmids in which the RTBV promoter was used to control expression of the effector genes, the enhanced 35S promoter was replaced by the full-length RTBV promoter (nucleotides −731 to +45) obtained from plasmid pMB9089 (12, 16).

Transient Expression Assays. Protoplasts isolated from suspension cultures of BY-2 cells (*Nicotiana tabacum* L., cv. Bright Yellow-2) were transfected via electroporation as described by Watanabe *et al.* (17). The protoplasts were cotransfected with a mixture of DNAs, including 5 μg of reporter gene construct, 20 μg of effector DNA construct, 5 μg of internal control plasmid containing the chimeric gene 35S::GFP, and 10 μg of herring sperm DNA by using a discharge of 500 μF and 250 V through disposable 0.4-cm cuvettes. Each transient assay was repeated three times per experiment and each experiment was conducted three times.

Plant Transformation. The plasmids containing the effector and reporter constructs were transferred by electroporation into *A. tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformation of *N. tabacum* cv. Xanthi NN was conducted as described (18). Kanamycin-resistant plants (R_0) were grown in the greenhouse and allowed to self-fertilize and R_1 seeds were collected. Plants carrying only a single insert of 35S::2C7-VP64 or C7F::G (Fig. 4*A*) were genetically crossed to obtain plants that contained both transgenes. Six R_0 lines were used in the crosses and R_1 seeds were collected. R_1 seeds were germinated in Murashige and Skoog medium (19) containing kanamycin 100 mg/liter, and seedlings that survived the selection were grown in soil in a greenhouse.

Analysis of Transgenic Plants. The protocol used to isolate genomic DNA from plants was a modified and simplified cethyltriethylammoniumbromide extraction method described by Futterer *et al.* (20). To assess the presence of transgenes, PCRs were used. The following oligonucleotides were used as primers: F (−18/+5), 5′-TTCGGCATTTGTGAAAACAAG-3′; β-glucuronidase (GUS)900–3′ (907/887), 5′-ACGTAAGTCCGCATCTTCAT3-′; GUS100–5′ (1693/1712), 5′-AACAAGAAAGGGATCTTCAC-3′; C7–3′ 5′-TTCAGATCAGCCGACTTAGA-

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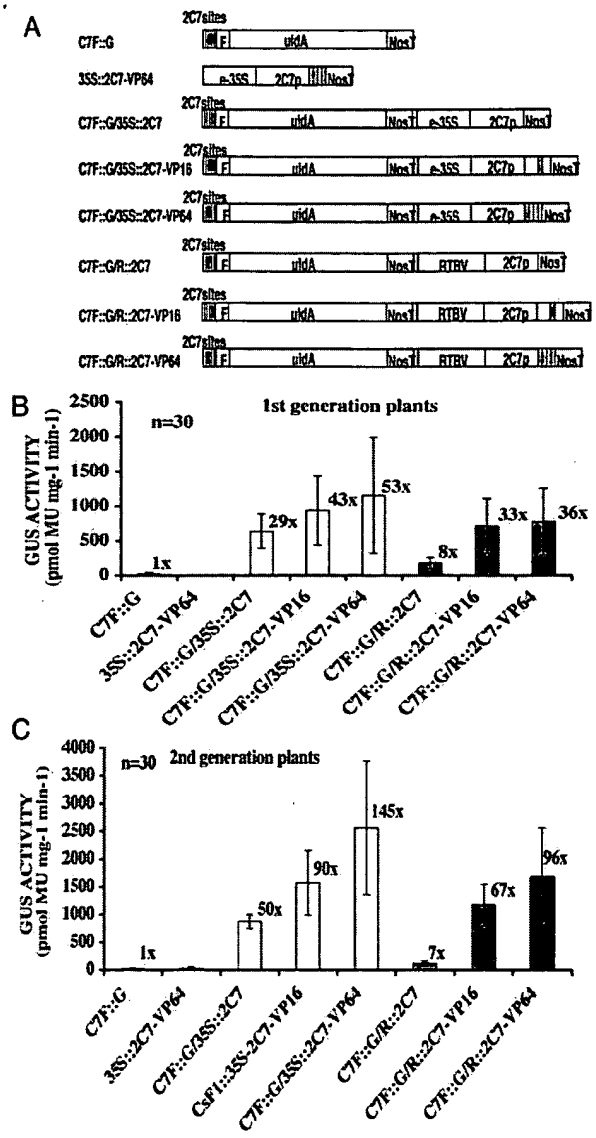

Fig. 3. Activation of GUS enzyme activity in R_0 and R_1 tobacco leaves using the F fragment of the CsVMV promoter (12). (*A*) Control and reporter–effector plant constructs used in the experiments. The control construct contains only the reporter gene, C7F::G or the TFs_{ZF} effector gene, 35S::2C7-VP64. In constructs that include both reporter and TFs_{ZF} effector genes in one plasmid, the effector genes are fused either to the enhanced 35S promoter or the RTBV promoter. (*B*) Measurement of GUS activity in leaf extracts from 30 R_0 tobacco plants containing the control or the reporter and effector constructs. Leaf extracts were taken for the assay 5 wk after the plants were potted in soil. PCR analysis was used to determine the presence of the reporter gene and the effector gene and to ensure that both genes were physically linked to each other. The results are expressed as amount of GUS enzyme activity (picomol MU $mg^{-1}{\cdot}min^{-1}$). The results are the average of GUS activity of 30 plants ± SD. The results are significantly different to C7F::G ($P < 0.05$). (*C*) GUS activity in R_1 tobacco plants. Seeds were collected from 15 R_0 plants, and 20 seeds of each were germinated on Murashige and Skoog medium supplemented with kanamycin. The results of GUS activity in leaf extracts are expressed as picomol MU $mg^{-1}{\cdot}min^{-1}$. The results presented are the average of GUS activity of the 20 plants ± SD ($P < 0.05$).

3′, VP16–3′, 5′-ATCTGCTCAAACTCGAAGTC-3′; VP64–3′, 5′-AATTAACATATCGAGATCGAAAT-3′; BoxII RTBV, 5′-CCAGTGTGCCCCTGG-3′, and 35S-53 5′-tgatcaaagctTATC-CTTCGCAAGACCCT-3′. We also confirmed that in plants

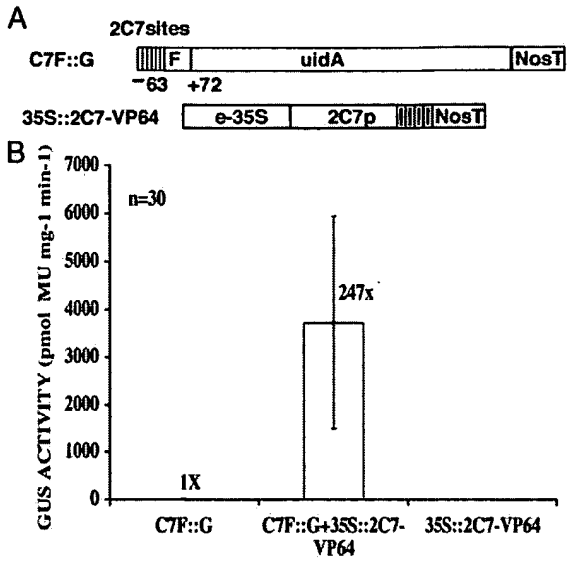

Fig. 4. GUS expression in leaves of the progeny of crossed tobacco plants transformed with the reporter construct C7F::G and the activator construct 35S::2C7-VP64. (*A*) Diagram of the constructs used in this experiment. The construct C7F::G (Fig. 1*A*) and the effector were each introduced to transgenic plants. (*B*) Quantitation of GUS activity in leaf extracts from 5-week-old seedlings of plants that contain the single genes or that contained both genes after crossing plants described in *A*. The results are the average of GUS activity (picomol MU $mg^{-1}{\cdot}min^{-1}$) of 30 plants ± SD ($P < 0.05$).

that carried both constructs on a single transgene the genes were physically linked to each other.

Fluorescence GUS Assay. Transfected protoplasts were lysed by freezing and thawing in GUS extraction buffer (pH 7.7; ref. 12) and centrifuged, and the supernatants were used for enzyme assays. GUS activity was determined by the method of Jefferson *et al.* (21). GFP activity was determined by quantifying fluorescence with 490-nm excitation wavelength and 530-nm emission wavelength (Molecular Devices). The concentration of proteins in cell extracts was determined by the dye-binding method of Bradford (22).

Histological GUS Assay. Explants sliced manually with a razor blade were stained with filter-sterilized 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) staining solution (50 mM phosphate buffer, pH 7.0/0.5 mM potassium ferricyanide/0.5 mM potassium ferrocyanide/0.2% Triton X-100/0.5% DMSO/20% methanol/2 mM EDTA/1 mM X-Gluc) overnight at 37°C. The staining was stopped, and chlorophyll was removed by incubating in 70% ethanol. Tissues were viewed with a Nikon Eclipse 800 with bright-field optics (×10, ×20 objectives).

Results

Transient Reporter Assays in BY-2 Protoplasts. *Transient expression using the CsVMV promoter.* To assess the efficacy of 2C7-based TFs_{ZF} in plant cells, we prepared three reporter and three effector gene constructs. The reporter constructs were built by using different fragments of the CsVMV promoter to drive expression of the *uidA* gene (encoding GUS): D (nucleotides −178 to +72); E (nucleotides −112 to +72); and F (nucleotides −63 to +72; ref. 12). In each case, six tandem repeats of the 18-bp DNA recognition site of the 2C7 protein was placed at the 5′ end of the promoter fragment. Effector protein (TFs_{ZF}) expression plasmids used the e-35S promoter (14) to drive

expression of the 2C7 protein alone (35S::2C7), the 2C7 protein fused at its C terminus with the herpes simplex VP16 transcription activation domain (ref. 11; 35S::2C7-VP16), or the 2C7 protein fused at its C terminal with the synthetic activation domain VP64 (35S::2C7-VP64; ref. 9; Fig. 1*A*). VP64 is a four times repeat of the minimal activation domain from VP16.

Three independent experiments were conducted, each with three samples per experiment. BY-2 protoplasts were electroporated to introduce reporter or/and effector constructs, and GUS activity was measured 24 h later by using 4-methyl umbelliferil glucuronide as a substrate (ref. 21; Fig. 1*B*). In preliminary experiments, we established that GUS activity was greatest at 24 h after transfection (data not shown).

The reporter construct C7D::G in the absence of cotransfected effector (Fig. 1*A*) was used as to normalize the effect of various TFs_{ZF} effector genes, including constructs 35S::2C7, 35S::2C7-VP16, and 35S::2C7-VP64 (Fig. 1*A*). We observed less than a 2-fold increase in GUS expression in protoplasts transfected with the C7D::G plasmid and any of the effector constructs (Fig. 1*B*). The C7E::G reporter gene (CsVMV nucleotides −112 to +72) was more responsive to the TFs_{ZF} than was C7D::G, and significant differences in GUS expression were observed with each TFs_{ZF}. Up to 7-fold enhancement of reporter gene activity was noted with TFs_{ZF} bearing the VP16 or VP64 activation domains. Similarly the C7F::G reporter construct was also responsive to expression of the TFs_{ZF}. In this case, reporter gene activity of the TFs_{ZF} 2C7-VP64 was significantly more potent than 2C7-VP16 (Fig. 1*B*). Similar results were obtained when reporter and effector genes were carried on a single plasmid or on two different plasmids (data not shown).

Transient expression using the RTBV and erbB-2 promoters. With the goal of reducing the basal level of reporter gene expression, we tested two other core promoters. The promoter from RTBV is a weaker promoter in BY-2 protoplasts as compared to the CsVMV promoter. In this case, we used a minimal RTBV promoter that contains the TATA box plus downstream sequences (T; nucleotides −32 to +45). This fragment lacks previously identified 5′cis elements (23) and was fused with the 2C7 recognition sites and the *uidA* reporter gene (C7T::G; Fig. 2*A*). The second construct included the TATA box region (er2; nucleotides −17 to +32) of the human *erbB-2* promoter with the 2C7-binding sites and the *uidA* gene (C7er2::G; Fig. 2*A*).

With the C7T::G reporter plasmid, we observed a 2.1-fold increase in activity with the 2C7-VP16 TFs_{ZF} and an average 4.7-fold increase with 2C7-VP64 (Fig. 2*B*). In studies involving the C7er2::G reporter gene plasmid, the TFs_{ZF} effector plasmid 35::2C7-VP16 increased GUS activity an average of 4.9 times, whereas the 2C7-VP64 TFs_{ZF} increased GUS levels 27-fold (Fig. 2*B*). This dramatic difference in GUS activity is due in part to the very low basal level of activity of the C7er2::G plasmid.

Expression in Tobacco Plants. To test the activity of the 2C7 TFs_{ZF} system in transgenic plants, the reporter construct C7F::G was introduced to transgenic plants alone, or with the effector constructs. The effectors genes produced either VP16 or VP64 and were driven either by the e-35S promoter (for constitutive expression) or the RTBV promoter (for phloem-specific gene expression). The constructs are designated, C7F::G/35S::2C7; C7F::G/35S::2C7-VP16; C7F::G/35S::2C7-VP64; C7F::G/R::2C7; C7F::G/R::2C7-VP16, and C7F::G/R::2C7-VP64 (Fig. 3*A*).

After transformation using *A. tumefaciens*, kanamycin-resistant plants were grown in a greenhouse and analyzed via PCR for presence of the reporter and effector genes. Thirty transgenic plants containing the target genes were selected for each gene construct, and extracts of fully expanded leaves were used for GUS enzyme assays. As shown in Fig. 3*B*, when the 2C7 protein was expressed under control of the e-35S promoter, GUS activity was increased an average of 29 times compared to plants that contained only the reporter gene. When the VP16 and VP64 activator domains were added to the 2C7 TFs_{ZF}, the amount of GUS activity increased an additional 1.5–1.9 times. When the RTBV promoter drove expression of the 2C7 gene, GUS activity increased an average of eight times, whereas the addition of the VP16 and VP64 activation domains increased GUS activity by an additional four times (Fig. 3*B*).

Fifteen of the 30 parental (R_0) transgenic plants lines with near average levels of GUS activity were allowed to self-fertilize, and their seeds were collected. The seeds were grown in Murashige and Skoog medium (19) supplemented with kanamycin. Twenty seedlings (R_1) were grown for 5 wk and leaf extracts were obtained, and GUS activity was determined (Fig. 3*C*). The average of GUS activity in plants that contained only the C7F::G transgene was set as one time. Plants that contained C7F::G/35S::2C7 had 50 times more GUS activity, and on addition of VP16 and VP64 domains to the zinc finger protein, the average GUS activity increased an additional 2–3 times, respectively. In the case of the RTBV promoter, the relative amount of GUS activity in plants with C7F::G/R::2C7 was seven times greater than in plants without the 2C7. Inclusion of the VP16 and VP64 domains increased GUS activity 8–14 times (Fig. 3*C*). The data collected from the R_1 generation of plants were consistent with the data from the parental R_0 lines; although the overall total amount of GUS activity was greater in the R_1 generation than in the R_0 generation of plants.

Binary transgene expression. The activity of the 2C7 TFs_{ZF} effector proteins was also tested in binary transgene expression studies. The reporter plasmid C7F::G and the TFs_{ZF} effector plasmid 35S::2C7-VP64 were introduced independently to transgenic plants. Six lines from each construct with an average GUS activity were selected for crossing and used as male and female parents (Fig. 4*A*). From each genetic cross, 15 seedlings were selected for further study after germination on Murashige and Skoog medium containing kanamycin and confirmation by PCR assays that they contain both plasmids. The results of GUS activity in the R_1 seedlings showed that plants that contain the TFs_{ZF} plus the reporter gene produced on average 250 times, and as much as 450 times, more GUS than plants containing only the C7F::G reporter plasmid (Fig. 4*B*).

Histochemical analysis. The distribution of GUS activity in plants that contained the single transgene constructs was analyzed (as in Fig. 3*A*). R_1 seedlings were selected on Murashige and Skoog medium supplemented with kanamycin then transferred to soil and grown in a greenhouse for 5 wk before analysis. Fresh tissue sections were obtained from the leaves, stems, and roots, and GUS activity was detected after exposing the tissues to 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), followed by the localization of indigo dye precipitates via light microscopy.

Plants containing the C7F::G reporter transgene did not contain detectable amounts of GUS activity in any tissue, as described for this promoter fragment (12) (Fig. 5*a 1–4*). When the plants contained the reporter plus effector transgenes C7F::G/35S::2C7, low amounts of GUS expression was detected in leaf, stem, and root. The levels of GUS expression were increased significantly by TFs_{ZF} containing the activation domains VP16 and VP64, as expected based on the results shown in Fig. 3*B* (Fig. 5*b 1–4*). By using the effector constructs that contained the RTBV promoter to drive the expression of the 2C7 gene, we did not detect GUS activity in any cells. In the presence of TFs_{ZF} containing the activation domains VP16 and VP64, GUS expression was detected in vascular tissues in leaves, stems, and roots (Fig. 5*c 1–4*).

Discussion

The goal of this study was to determine the effectiveness of artificial TFs_{ZF} to control gene expression in transgenic plants

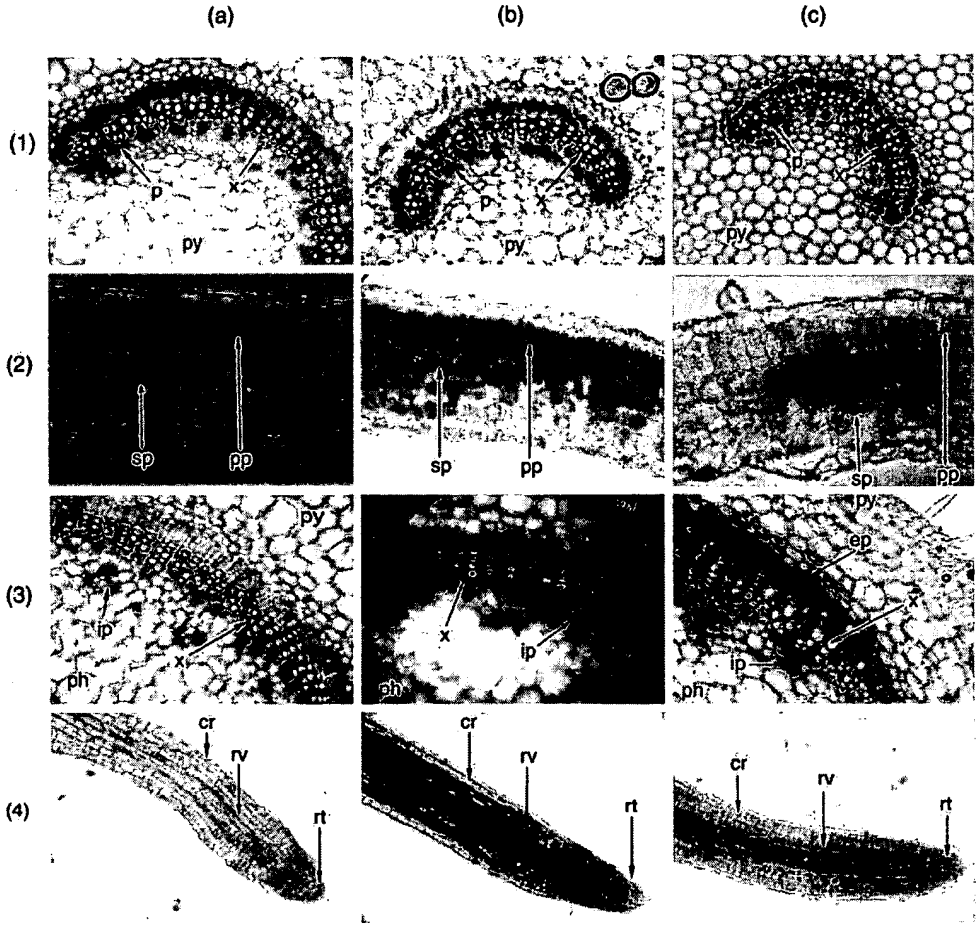

Fig. 5. Histochemical localization of GUS in transgenic tobacco plants containing: the C7F::G construct (*a*), C7F::G/35S::2C7-VP64 (*b*), and C7F::G/R-2C7-VP64 (*c*) (constructs as in Fig. 3). (*1*) Cross section of leaf showing the vascular tissue of the midrib. (*2*) Transverse section of leaf lamina. (*3*) Cross section of stem tissue. (*4*) Whole roots. GUS activity was localized after staining of tissue slices with X-Gluc and visualizing the indigo dye precipitates formed via light microscopy. cr, cortex root; ep, external phloem; ip, internal phloem; p, phloem; ph, pith; pp, palisade parenchyma; py, parenchyma; rt, root tip; rv, root vein; sp, spongyll parenchyma; and x, xylem.

and plant cells. The six-zinc finger protein system is based on specific interactions among 18 contiguous nucleotides (in this study the 2C7-binding site) and the six-zinc finger protein (the 2C7-binding protein). High theoretical specificity in sequence recognition is a key feature of these proteins and increases the potential that zinc finger proteins can be used to regulate the expression of target genes with minimal side effects. Artificial zinc finger proteins have been developed for use in animal and human cells and results of studies to date indicate a high potential for applications of this approach in gene therapy (7–11).

We first tested the efficacy of the 2C7 TFs_{ZF} system in tobacco BY-2 protoplasts as a predictor of their activity in whole plants. In this study, we used several different promoters to drive the production of the 2C7 TFs_{ZF} effector proteins and determined the role of the effectors on several reporter constructs. Reporter genes were derived from the CsVMV promoter (12), including fragment D (nucleotides −178 to +72), E (nucleotides −112 to +72), and F (nucleotides −63 to +72). In each case the 2C7 DNA-binding site was placed at the 5′ end of the promoter fragment.

The C7F::G reporter gene yielded low levels of GUS activity, and cotransfection of the 2C7 TFs_{ZF} protein as a fusion with the activation domains VP16 and VP64 resulted in approximately five times higher levels of GUS activity (Fig. 1*B*). C7D::G showed a lower degree of enhancement by the 2C7 TFs_{ZF} proteins, whereas C7E::G was enhanced by approximately the same proportion as C7F::G (comparing expression enhanced by 35S:: 2C7 vs. 35S::2C7-VP64). Differences in activation by TFs_{ZF} of the reporter genes may be caused by the differences in distance between the promoter and the 2C7-binding site. Another possible explanation is the presence of other cis elements in the longer promoters (C7D::G and C7E::G); binding of additional factors may affect the final level of gene expression (12).

We also studied the regulation of two different minimal promoters, C7T::G and C7er2::G. The C7T::G promoter, derived from the phloem-specific promoter from Rice Tungro Bacilliform Virus (nucleotides −32 to +45), has very low activity in both protoplasts and transgenic plants. In the presence of TFs_{ZF} there was a 4–5 times increase in GUS activity. The C7er2::G promoter, which is derived from the human erbB-2 promoter (9), did not produce detectable amounts of GUS activity in protoplasts. As a consequence, there were high levels of activation when 2C7 TFs_{ZF} proteins with activation domains were cointroduced with the reporter genes (5 and 27 times, respectively). These results are similar to those obtained when

the 2C7 TFs_{ZF} were used in mammalians cells with the full-length erbB-2 promoter (9).

The 2C7 TFs_{ZF} effector proteins were also active in transgenic tobacco plants. In lines that contain the 2C7 TFs_{ZF} under the control of the e-35S promoter, the addition of the activation domains increased expression of C7F::G between 1.5 and 2 times above the levels of the 2C7 protein alone (Fig. 3*B*). This is similar to increases in expression in BY-2 protoplasts (Fig. 1*B*). In the R_1 generation, the range of additive effects of the activation domains was somewhat greater, and up to a 145 times increase in gene expression was observed. It is important to note that the 2C7 TFs_{ZF}, in the absence of VP16 or VP64 domains, caused a significant increase (≈50 times) in GUS activity (Fig. 3*C*). These effects can be a result of changes in chromatin structure because of binding of 2C7 *per se*, that effects the binding of other factors to the DNA. It is known that local changes in chromatin structure brought about by transcription factor binding can lead to changes in gene expression (24).

When the reporter gene C7F::G and the TFs_{ZF} effector gene 35S::2C7-VP64 were introduced in separate plants, and the R_0 progeny of 12 plant lines were crossed, there was an average of ≈250 times activation of the reporter gene and a maximum enhancement of ≈450 times. This is twice the level of enhancement that was observed when the reporter and effector genes were introduced on a single plasmid.

The C7F::G reporter gene did not produce GUS activity in transgenic tobacco plants (Figs. 3 and 5; ref. 12), unless the 2C7 TFs_{ZF} proteins were coexpressed. When the constitutive e-35S promoter (21) was used to produce the TFs_{ZF} proteins, GUS activity was detected in all plant tissues. Similarly, when the phloem-specific RTBV promoter (13, 16) was used, GUS activity was restricted to the vascular system. Thus, expression of the reporter gene is controlled by the pattern of expression of the genes encoding TFs_{ZF}.

Based on our studies, we conclude that the degree of control of gene expression by synthetic zinc finger proteins will depend on the position of the binding site relative to the TATA box (or other sequences to which RNA pol II binds) and the inherent activity of the promoter that is targeted. In an accompanying paper, Guan *et al.* (25) report that a synthetic zinc finger that was designed to bind to endogenous DNA sequence 5′ of the *Apetala3* gene in *Arabidopsis* exerted control of expression of this gene and changed the phenotype of the transgenic plant line. Taken together with the results reported here, we conclude that synthetic proteins can be used to manipulate the expression of foreign as well as endogenous genes and will have applications in agricultural biotechnology.

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1. Orphanides, G. & Reinberg, D. (2002) *Cell* **108,** 431–451.
2. Somia, N. V., Kafri, T. & Verma, I. M. (1999) *Nat. Biotechnol.* **17,** 224–225.
3. Fisher, J. A., Giniger, E., Maniatis, T. & Patshne, M. (1988) *Nature (London)* **322,** 853–856.
4. Hartley, K. O., Nutt, S. L. & Amaya, E. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 1377–1382.
5. Ma, J., Przbilla, E., Hu, J., Bogorad, L. & Patshne, M. (1988) *Nature (London)* **334,** 631–633.
6. Riechmann, J. L. & Ratcliffe, O. J. (2000) *Curr. Opin. Plant Biol.* **3,** 423–434.
7. Segal, D. J. & Barbas, C. F., III (2001) *Curr. Opin. Biotechnol.* **12,** 632–637.
8. Beerli, R. R. & Barbas, C. F., III (2002) *Nat. Biotechnol.* **20,** 135–141.
9. Beerli, R. R., Segal, D. J., Dreier, B. & Barbas, C. F., III (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14628–14633.
10. Beerli, R. R., Dreier, B. & Barbas, C. F., III (2000) *Proc. Natl. Acad. Sci. USA* **97,** 1495–1500.
11. Liu, Q., Segal, D. J., Ghiara, J. B. & Barbas, C. F., III (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5525–5530.
12. Verdaguer, B., de Kochko, A., Fux, C. I., Beachy, R. N. & Fauquet, C. (1998) *Plant Mol. Biol.* **37,** 1055–1067.
13. Yin, Y. & Beachy, R. N. (1995) *Plant J.* **7,** 969–980.
14. Kay, R., Chan, A., Daly, M. & McPherson, J. (1987) *Science* **236,** 1299–1302.
15. Hajdukiewicz, P., Svab, Z. & Maliga, P. (1994) *Plant Mol. Biol.* **25,** 989–994.
16. Bhattacharyya-Pakrasi, M., Peng, J., Elmer, J. S., Laco, G., Shen, P., Kaniewska, M. B., Kononowicz, H., Wen, F., Hodges, T. K. & Beachy, R. N. (1993) *Plant J.* **4,** 71–79.
17. Watanabe, Y., Meshi, T. & Okada, Y. (1987) *FEBS Lett.* **219,** 65–69.
18. Horsch, R. B., Fry, J., Hoffman, N., Neidermeyer, J., Rogers, S. G. & Fraley, R. T. (1988) in *Plant Molecular Biology Manual*, eds. Gelvin, S. B. & Schilperoort, R. A. (Kluwer, Dordrecht, The Netherlands), pp. A5/1–A5/9.
19. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15,** 473–497.
20. Futterer, J., Gisel, A., Iglesias, V., Kloti, A., Kost, B., Mittelsten-Scheid, O., Neuhaus, G., Neuhaus-Url, G., Schrott, M., Shillito, R., *et al.* (1995) in *Gene Transfer to Plants*, ed. Spangenberg, G. (Springer, Berlin), pp. 215–218.
21. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. (1987) *EMBO J.* **6,** 3901–3907.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
23. Yin, Y., Chen, L. & Beachy, R. (1997) *Plant J.* **12,** 1179–1188.
24. Vashee, S., Willie, J. & Kodadek, T. (1998) *Biochem. Biophys. Res. Commun.* **247,** 530–535.
25. Guan, X., Stege, J., Kim, M., Dahmani, Z., Fan, N., Heifetz, P., Barbas, C. F., III, & Briggs, S. P. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 13296–13301.